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Award Number: DAMD17-02-1-0426

TITLE: HOXB7: An Oncogenic Gene in Breast Cancer Cells?

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REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20031028 144

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2003		3. REPORT TYPE AND DATES COVERED Annual Summary (15 Apr 02 - 14 Apr 03)
4. TITLE AND SUBTITLE HOXB7: An Oncogenic Gene in Breast Cancer Cells?			5. FUNDING NUMBERS DAMD17-02-1-0426	
6. AUTHOR(S) Ethel Rubin, Ph.D. Saraswati Sukumar, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University School of Medicine Baltimore, Maryland 21205 E-Mail: emrubin@jhmi.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Homeobox genes control anterioposterior body axis patterning during development. Although expressed primarily in developing embryos, a growing body of evidence shows that homeobox gene re-expression in adult tissues is associated with tumorigenesis. Earlier work has shown that introduction of HOXB7, one of the members of this gene family, into non-expressing breast cancer cells induced expression of a number of pro-angiogenic factors and formed tumors upon xenograft into nude mice. We had earlier identified HOXB7 as an overexpressed gene in a SAGE analysis of breast cancer cell lines and wanted to investigate its oncogenic potential in this study. To characterized the role of HOXB7 in breast cancer, a number of HOXB7-interacting proteins in breast cancer cells and HMECs were identified by a combination of mass spectrometry and direct sequencing techniques. Co-immunoprecipitation experiments confirmed the interaction in vivo. Interestingly, all of the HOXB7-associated proteins identified have well characterized roles in the non-homologous end joining (NHEJ) pathway for DNA double strand break (DSB) repair as well as a larger role in maintenance of genomic integrity. Cell survival and chromosomal analysis experiments deomonstrate that breast cancer cells stably transfected with HOXB7 survive better after induction of DNA DSB with fewer chromosomal abnormalities following exposure to gamma radiation. These data have led us to propose that HOXB7 may have a role in DNA DSB repair and that HOXB7 may provide a survival advantage for tumors which express this gene.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

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Introduction

Class I homeobox genes are transcription factors which are involved in anterioposterior body axis positioning during development in vertebrates. There are 39 mammalian HOX genes clustered on human chromosomes 2, 12, 7 and 17 (1, 2). Genes at the same relative position harbor the greatest sequence similarity and are thus known as paralogs. All HOX genes bind to similar core DNA motifs through their homeodomains (3, 4, 5), though sequence specificity may be determined by regions flanking the DNA consensus site, or by HOX binding proteins which are postulated to play a major role in sequence specific DNA binding and transcriptional activation (6-12). To date, few HOX binding proteins have been found, yielding great impetus to elucidate the regulation of HOX-mediated gene expression.

We recently performed a SAGE analysis of breast cancer cell lines which identified a number of differentially expressed genes that may play a role in the disease course (13). Of those, two were HOX genes. While HOXA5 expression was lost in the breast cancer cell lines, that of HOXB7 was augmented compared to HMEC. HOXB7 overexpression may be due to gene amplification (14) and is correlated with poor patient prognosis. When transduced into breast cancer cells, HOXB7 expression has been shown to affect tumor formation in nude mice, and its downstream targets appear to be both mitogens such as bFGF as well as pro-angiogenic factors (15, 16, 17), indicative of an oncogenic role in breast cancer. To uncover the role that HOXB7 may be playing in breast cancer, and further the understanding of HOX protein function, we identified a number of HOXB7-binding proteins from mammary epithelial cells. Interestingly, the HOXB7 interacting proteins all fell into a group of genomic caretakers, including members of the NHEJ pathway of DNA DSB repair, and poly-ADP ribose polymerase (PARP), which have roles in the maintenance of genomic integrity.

Maintenance of genomic integrity relies on both faithful DNA repair mechanisms as well as eradication of cells carrying cancer-causing changes or mutations. DNA DSB repair in mammalian cells occurs by two major pathways: homologous recombination and nonhomologous end joining (NHEJ). In NHEJ Ku80 together with its heterodimeric binding partner Ku70, are subunits of a DNA-dependent protein kinase holoenzyme (18, 19, 20). Ku 80 is thought to recruit the enzymes required for repair of the DS ends, including the catalytic subunit of DNA-PK, XRCC4 and ligase IV(21). The DNA-PK also plays a major role in immunoglobulin VDJ recombination (22) and as such, Ku 70 and 80 have been shown to bind to numerous nuclear proteins both as a complex and independently (23-28). While acting as a DS DNA end binding protein, Ku 80 also associates with telomeres and telomere binding proteins (29, 30, 31) where it is thought to prevent inappropriate recognition of these DS ends by the NHEJ machinery and is postulated to restrict access of telomerase to the DNA ends as Ku 80 knockout mice possess elongated telomeres (32-35).

PARP is an enzyme family consisting of 4 members which catalyze the transfer of ADP-ribose from an NAD substrate onto protein targets, including histones, transcription factors and self (36-38 for review). Although present at a basal level in most cells, this activity is stimulated by DNA damage or other forms of genotoxic stress. In response to DNA damage, transcription factors which become ADP-ribosylated carry a high net negative charge, causing electrostatic repulsion between protein and DNA. DNA binding is abolished, allowing the necessary DNA repair to take place before further rounds of transcription. PARP activity is necessary for a caspase-independent apoptotic pathway through its target AIF (39), again, a mechanism for maintenance of genomic integrity. Although there is some controversy regarding the telomeric maintenance of cells derived from PARP knockout mice (40, 41), PARP is required for maintenance of telomeric ends.

The finding that HOXB7 was involved in interactions with members of the DNA-PK holoenzyme and PARP led us to examine whether HOXB7 expression affects NHEJ, and is involved in the maintenance of genomic integrity. Our results show that HOXB7 overexpression in breast cancer cells stimulates NHEJ *in vitro* and that both DNA repair and telomeric stability are enhanced *in vivo* in cells engineered to overexpress HOXB7. Taken together, it appears that breast cancer cells which have re-expressed HOXB7 have a survival advantage which sustains their oncogenic program.

To determine the oncogenic potential of HOXB7 in mammary epithelial cells, we overexpressed HOXB7 in the normal mammary epithelial cell line, MCF10A and tested transformation *in vitro* in soft agar growth assays. In comparison with vector transfected controls, HOXB7-MCF10A cells formed numerous colonies containing many cells. To test whether this transformation capability required the interaction of HOXB7 with its binding partners, the soft agar growth assay was repeated in the presence of a potent PARP inhibitor, 3-aminobenzamide. In the presence of this inhibitor, HOXB7-MCF10A colony formation on soft agar was reduced four-fold, while no effect was seen with a PARP inhibitor analog compound. While many of these experiments are ongoing, and we are further testing the requirement for protein-protein interaction in HOXB7 transformation, the data thus far has lead us to propose that HOXB7 transforms cells by virtue of its interactions with caretaker proteins such as PARP.

2003 Annual Summary Report

Body

The overall goal of this study was to determine the oncogenic potential of HOXB7, to identify its target genes and interacting proteins for clues as to the mechanism of transformation, and to determine if HOXB7 could serve as a detection marker for diagnosis of breast cancer. Towards these ends, much progress has been made, as indicated in the list of key accomplishments, as well as below.

Task 1 of the Statement of Work investigates the transforming potential of HOXB7 *in vitro* by soft agar growth assays. MCF10A normal mammary epithelial cells and SKBR3 breast cancer cells were stably transfected with FLAG-tagged HOXB7 expression constructs or empty vector controls. In addition, HOXB7-YFP fusion constructs were made and stably transfected into SKBR3 breast cancer cells. When tested for transformation *in vitro* by growth on soft agar, only HOXB7-expressing MCF10A cells formed colonies. MCF10A cells or vector-transfected controls form little to no colonies on soft agar. While SKBR3 parental and vector-transfected control cells do form colonies on soft agar, there was a 33% increase in the number of colonies which form when these cells express HOXB7-YFP. These data support the hypothesis that HOXB7 has transformation capabilities *in vitro*.

Task 2 of the Statement of Work is to identify HOXB7 target genes by microarray and interacting proteins. While the target gene analysis and validation has not yet been performed, much progress has been made in identifying HOXB7 interacting proteins and characterizing elements of these interactions. The next few paragraphs will describe progress made in this area.

Identification of HOXB7-binding proteins

GST-pulldown assays were utilized to discover HOXB7 binding proteins. When GST-HOXB7 was mixed with extracts of SKBR3 breast cancer cells, which do not express HOXB7, 4 unique protein bands at mw 65, 85, 110, and approximately 300 kD were evident on silver-stained gels. Neither GST alone nor GST-PRL3, an unrelated GST fusion protein, bound these proteins. Identical pulldowns were performed with extracts of MCF10A, a normal immortalized mammary epithelial cell line, and MDA-MB-231 breast cancer cells with the same results. To determine the identity of these proteins, a combination of direct amino acid sequencing as well as peptide mass fingerprinting were used. N-terminal amino acid sequencing identified the 85kD band to be Ku 80, the human DNA repair gene XRCC5. Peptide mass fingerprinting was employed for identification of the 65 kD and 110 kD bands, which appeared to match peptide mass maps of Ku 70 and Poly-ADP-ribose polymerase (PARP), which respectively have masses of 70 and 113 kD. Since Ku 70 and 80 form a heterodimeric subunit for DNA-PK, the 300 kD protein band was thought to possibly be the catalytic subunit of DNA-PK (DNA-PKcs). Indeed, western blot analysis of the GST-pulldown assay confirmed the identity of DNA-PKcs as well as the rest of the HOXB7-binding protein bands. Ku 70 and 80 together with DNA-PKcs form the DNA-PK holoenzyme which plays a major role in double strand (DS) DNA repair by the non-homologous end joining pathway (NHEJ).

HOXB7 binds to Ku, DNA-PK and PARP in vivo

To confirm that the interactions of HOXB7 with Ku/DNA-PK and PARP mirrored the situation *in vivo*, co-immunoprecipitation experiments were performed. HOXB7 was stably transfected into SKBR3 cells in a YFP fusion in order to visualize HOXB7 *in vivo*. The HOXB7-YFP localized to the nucleus in confocal-microscopy images while that of the vector-transfected

SKBR3 controls was dispersed throughout the cell. When the HOXB7-YFP transfected cells were subject to immunoprecipitation with GFP antibodies, Ku 70, 80, DNA-PKcs and PARP were all co-immunoprecipitated. Control immunoprecipitations, and vector-transfected cell controls did not co-precipitate significant amounts of these proteins. Complimentary immunoprecipitations performed with antibodies to Ku80 and DNA-PKcs also co-precipitated HOXB7-YFP but not YFP alone. Furthermore, the interaction of HOXB7 and Ku 70, 80, DNA-PKcs and PARP was observed in FLAG antibody immunoprecipitates of FLAG-HOXB7 transfected cells. These results confirmed our *in vitro* data showing interaction between HOXB7 and DNA-PK as well as PARP.

DNA binding is not required for the interaction

To ensure that the interaction was not mediated by virtue of DNA binding, GST pulldown assays and co-immunoprecipitation experiments were performed in DNase 1-treated cell extracts. GST-HOXB7 was treated with DNase 1-treated to eliminate bacterial DNA that may co-purify with the recombinant protein prior to mixing with DNase-treated cell extracts. Complex formation between GST-HOXB7 and Ku 70, 80, DNA-PKcs and PARP was maintained under conditions in which DNA was absent. The interaction was also examined *in vivo* by co-immunoprecipitating FLAG-HOXB7 with its interacting proteins from cell extracts treated with DNase 1. Again, the absence of DNA did not affect the interaction. This confirmed that the interaction was not mediated by DNA binding.

HOXB7 serves as a substrate for DNA-PK phosphorylation and PARP-mediated ADP-ribosylation

To determine if HOXB7 could be a substrate for the kinase activity of DNA-PK, GST-HOXB7 was mixed with purified DNA-PK in the presence of γ -³²P-ATP. In the presence of active DNA-PK, GST-HOXB7 was effectively phosphorylated while GST was not. In attempting to determine if phosphorylation of HOXB7 would affect its DNA binding function, we have run into technical difficulty. Gel shift analysis was performed to determine if phosphorylated HOXB7 could bind a DNA oligomer containing a HOXB7 consensus binding site (16). Upon first trial, no DNA binding was apparent for either unphosphorylated nor phosphorylated GST-HOXB7. While this DNA oligomer originated from the bFGF promoter, which HOXB7 has been shown to bind and activate transcription, it apparently is not bound by GST-HOXB7. It is possible that binding of recombinant HOXB7 will not occur in the absence of PBX1, a transcriptional partner of HOX proteins. We will repeat this experiment mixing recombinant PBX1 into the binding reaction, use other published consensus sequences, or other HOXB7-binding sequences we find as a result of our microarray analysis.

To determine if HOXB7 could be ADP-ribosylated by PARP, we mixed GST-HOXB7 or GST alone with purified PARP in the presence of ³²P-NAD. The reaction was loaded onto SDS-PAGE gels which were dried and exposed to film. GST-HOXB7 was efficiently ADP-ribosylated by PARP *in vitro*. To determine if HOXB7 could serve as a substrate for PARP *in vivo*, SKBR3 cells stably expressing HOXB7-YFP or FLAG-HOXB7 were permeabilized in the presence of ³²P-NAD. The cells were lysed and the HOXB7 was immunoprecipitated. Protein complexes were resolved by gel electrophoresis, transferred to nitrocellulose and exposed to film. After autoradiography, in which only a single band appeared, the nitrocellulose blots were probed for HOXB7 using either YFP or FLAG antibodies. The bands that were labeled with ³²P aligned with the HOXB7 specific bands. No bands appeared in vector-transfected control cell

immunoprecipitates, nor in reactions in which cells were pre-incubated with a PARP inhibitor. Since PARP transfers poly-ADP-ribose moieties to lysine, aspartic and glutamic acid residues on protein targets, we hypothesized that the glutamic acid-rich carboxy-terminal tail of HOXB7 was the acceptor site for the ADP-ribose. To test this, the *in vivo* PARP assay above was repeated in SKBR3 cells transiently transfected with a FLAG-HOXB7 mutant lacking this glutamic acid tail. While western blots showed that this protein was precipitated during the experiment, no ^{32}P incorporation was apparent. Thus HOXB7 is ADP-ribosylated by PARP *in vitro* and *in vivo* and the site of ADP-ribosylation appeared to be the glutamic acid tail at its carboxy terminus.

PARP and Ku make distinct complexes with HOXB7

Ku 70 and 80 form a well-characterized heterodimer in human and yeast cells. PARP has also been shown to bind to Ku 80 (44). Therefore, in order to characterize which protein makes distinct complexes with HOXB7, Chinese Hamster ovary cells (CHO), which do not contain Ku 70 nor Ku80 (and have much less PARP than human cells) were utilized. FLAG-HOXB7 was transfected into CHO cells alone or together with different combinations of PARP, Ku70 and Ku 80. While HOXB7 co-immunoprecipitated with PARP in the absence of Ku 70 and 80, Ku 70 and 80 were both required for interaction with HOXB7. Thus the Ku subunits require prior heterodimerization for interaction with HOXB7 while PARP binds HOXB7 independently of its interaction with Ku80.

The Homeodomain mediates the interaction

To determine which region of HOXB7 mediates the interaction with PARP and Ku proteins, FLAG-tagged HOXB7 mutants which were constructed by Yaron et. al. (42) were utilized. SKBR3 and CHO cells were transiently transfected with these HOXB7 mutant constructs and binding to PARP and Ku proteins was tested by co-immunoprecipitation. While all mutants bound to PARP and Ku 70 and 80, there was significantly less binding apparent for the HOXB7 mutant lacking the third helix of the homeodomain. We thus propose that the homeodomain of HOXB7 mediates its interactions with PARP and Ku 70/80 heterodimers.

Effects of PARP and Ku on HOXB7 transcriptional activity

While we are interested in discovering the effects of HOXB7 binding proteins on its transcriptional activity, we have again run into technical difficulties. The only known HOXB7 target gene which has been characterized to sufficient extent is that of bFGF. When tested for transcriptional activity by transient transfection assays, both by luciferase or CAT reporter assays, no effect was evident whether or not PBX1 was co-transfected with HOXB7 expression constructs in a number of different cell lines, including SKBR3, MDA-MB-231, CHO, MCF7 and NIH-3T3. We therefore hope that our microarray analysis to discover HOXB7 targets will yield genes whose promoters can be used to test HOXB7 transcriptional activity and its modulation by its interacting proteins.

The effect of HOXB7 on NHEJ and telomeric stability

Since HOXB7 interacts *in vitro* and *in vivo* with proteins that are part of the NHEJ pathway of DNA double strand break (DSB) repair and genomic maintenance, we tested whether HOXB7 overexpression affects these processes. First, we tested whether HOXB7-containing nuclear extracts would affect an artificial DSB substrate. We mixed HOXB7-containing or vector transfected control nuclear extracts *in vitro* with DS DNA plasmids that were linearized (both blunt-ended and with overhangs) by restriction and then gel-purified. Reactions were resolved

on 0.7% agarose gels and visualized by ethidium bromide staining. While all nuclear extracts had plasmid-end joining activity, that of the HOXB7-containing cells was enhanced by 30-40% as determined by comparison of densitometric values of end-joined plasmid products. Next, HOXB7-YFP expressing SKBR3 cells were irradiated with low dose gamma radiation which induces DNA DSB. When the relative survival of these cells or vector-transfected controls were measured, that of the HOXB7 expressing cells was higher by about 30%. The mitotic index of these cells was higher following DNA damage and when either G1 or G2 type chromosomal damage was examined following low-dose irradiation, the HOXB7-YFP expressing cells had significantly less damaged chromosomes both 45 and 90 minutes following induction of DNA DSB. These data indicate that in cells overexpressing HOXB7-YFP, DNA DSB repair occurred much faster than in parental or control cells. (Although chromosomal repair appeared to be enhanced, this should not be confused with correct maintenance of genomic integrity, which requires faithful sequence maintenance. In fact, the NHEJ pathway is associated with increased mutagenicity (45)). Telomeric maintenance was examined in HOXB7-YFP expressing, vector control and parental cells by fluorescent in-situ hybridization (FISH). Only HOXB7-expressing cells had every chromosome in the spread fluorescently labeled, indicative of enhanced telomeric stability. These data lead us to propose that HOXB7-overexpressing cells have enhanced DNA repair and survival abilities *in vivo*, with enhanced replicative potential, as appropriate for oncogenic transformation capabilities of HOXB7.

Is the HOXB7 transformation capability dependent on its protein-protein interactions?

The question remains if HOXB7 transformation requires its protein-protein interactions. To begin to test this question, we repeated the soft agar growth *in vitro* transformation assay in the presence of a potent PARP inhibitor, 3-aminobenzamide (3-ABA), or its non-PARP inhibiting analog (aminobenzoic acid). HOXB7-MCF10A cells or vector-transfected controls were grown on soft agar in the presence or absence of 7 mM 3-ABA or its non-inhibitory analog. Much to our surprise, the number of colonies was reduced 4-fold in the presence of the PARP inhibitor but not in its analog control. We did not have a negative control cell line which would form colonies on soft agar but would not be affected by a PARP inhibitor to test at the time of the experiment but have recently received such transfected MCF10A cells from a colleague and will repeat this assay. We also would like to test whether the interaction with PARP would affect HOXB7 transformation in a second way, by PARP inhibition via siRNA. To do so, siRNA for PARP will be introduced into HOXB7-MCF10A and vector-transfected control cells and the soft agar assay will be repeated if abrogation of PARP expression in this way is successful. To test if the interaction with Ku 70 or 80 mediates HOXB7 transformation, siRNA for Ku70 and Ku80 will be introduced into the HOXB7-MCF10A and vector transfected control cells and the soft agar assay will be repeated as well. While also important, we are more concerned with determining if the mechanism of HOXB7 transformation is through its interaction and ADP-ribosylation by PARP. In a recent publication, PARP has been shown to regulate centrosome copy number and coupling of centrosome duplication with the cell cycle. PARP binds to centrosomal proteins and transfers ADP-ribose moieties to these endogenous substrates, regulating their activity. This process is crucial in maintaining genomic integrity of cells, as dysregulated centrosomes leads to malfunction of sister chromatid separation during mitosis, resulting in many of the ploidy phenotypes observed in cancer cells (43). Since we have shown that HOXB7 is ADP-ribosylated *in vivo* by PARP, does it act as a competitor for other endogenous PARP substrates, affecting their regulation by ADP-ribosylation? We would like to test this in ADP-ribosylation assays in HOXB7-MCF10A or control cells. Does tubulin (a centrosomal protein) become equally ADP-ribosylated when HOXB7 is present in cells? We

will visualize centrosomes in HOXB7-MCF10A and control cells by immunofluorescence staining and determine if there are altered copy numbers. We hypothesize that endogenous PARP substrates, such as centrosomal proteins, have diminished ADP-ribosylation levels if HOXB7 is overexpressed, but not if a mutant form lacking the glutamic acid carboxy-terminal tail. If so, it is possible that the mechanism for HOXB7 transformation is by virtue of competition for ADP-ribosylation and PARP activity, resulting in dysregulated centrosomal function and maintenance of genomic integrity which is a hallmark of cancer cells.

Task 3 of the Statement of Work aimed to develop HOXB7 as a detection marker for breast cancer diagnosis. Towards this end, we raised and purified a peptide-directed HOXB7 specific polyclonal antibody. The antibody was tested for specificity by western blot and did not detect a number of HOX proteins nor the related PBX1 protein produced by in vitro transcription/translation. It was highly specific for HOXB7. In order to test for the presence of HOXB7 that may be shed into the blood of breast cancer patients, ELISA assays were performed. We could not detect any difference between normal volunteer and breast cancer patient serum for reactivity. We also tested whether breast cancer patients had circulating antibodies for HOXB7. To do this, GST-HOXB7 was expressed in bacterial cells and purified by affinity chromatography. 100 ng of this purified recombinant protein was plated in each well of an ELISA plate. Serial dilutions of 16 patient sera (range was from 1:100 to 1:10000) and 14 normal volunteers was used to test for reactivity. Both anti-GST and the HOXB7 antibodies were used as positive controls, with normal rabbit serum as the negative control. No differences in reactivity were apparent between normal volunteer and breast cancer patient sera. Although possessing high specificity, the HOXB7 specific antibody was found to have low affinity, able to detect only high levels of HOXB7 from cells. We therefore are trying again, and in collaboration with Zymed Laboratories, S. San Francisco, CA, are developing new antibodies to HOXB7 and will re-test the patient serum for circulating antibodies to this protein.

Key Research Accomplishments

Task 1: Investigate the transforming potential of HOXB7 *in vitro* (months 1-12)

- Stably expressed HOXB7 and vector control plasmid in MCF10A normal mammary epithelial cells
- Stably expressed HOXB7-YFP and vector control plasmid in SKBR3 breast cancer cells
- Showed that HOXB7-expressing cell lines (above) formed more colonies in soft agar assays than controls

Task 2: Identify HOXB7 target genes and interacting proteins (months 13-24)

- Identified four HOXB7-interacting genes by peptide mass fingerprinting following GST-pulldown assays
- Confirmed and characterized the interaction *in vivo* in co-immunoprecipitation experiments
- Discovered two previously uncharacterized post-translational modifications of HOXB7
- Identified effects of HOXB7 on DNA double strand break repair *in vitro* and *in vivo*
- Found effects of HOXB7 overexpression on telomere stability *in vivo*
- Attenuated HOXB7-mediated transformation by PARP inhibition

Task 3: Develop HOXB7 as a detection marker for diagnosis (months 1-36)

- Expressed and purified recombinant GST-HOXB7 for use in ELISA assays
- Raised HOXB7-specific polyclonal antibodies

List of reportable outcomes

1. Minisymposium presentation

American Association of Cancer Research - July 2003

Physical and Functional Interaction of HOXB7 with members of the NHEJ pathway in Breast Cancer

Ethel Rubin, Ph.D.

2. Rubin, E., H. Chen, G. Sharma, S. Dhar, T.K. Pandita, and S. Sukumar. 2003.

Physical and functional interaction of HOXB7 with members of the NHEJ pathway in breast cancer. Proceedings of the American Association of Cancer Research, 44, Abstract 902

3. Poster Presentation

Novel Functions for HOXB7 in Breast Cancer.

E. Rubin, G. Sharma, S. Dhar, T.K. Pandita and S. Sukumar.

The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Fellow Research Day, April 17 2003

Conclusions

During the course of this study, a number of stably-transfected cell lines were established, both normal mammary and breast cancer, that overexpress HOXB7 in fusion with either YFP or FLAG to facilitate detection and visualization in living cells. We have used these cells to measure HOXB7-dependent transformation as well for identification of HOXB7-interacting proteins. Our results indicated that, as expected, HOXB7 expression was required for the normal immortalized mammary epithelial cell line, MCF10A, to grow on soft agar in an *in vitro* transformation assay. From our interaction experiments, four proteins were found to bind to HOXB7 *in vitro* and *in vivo*. Of these, three are part of the multicomplex assembly that is the DNA-dependent protein kinase, DNA-PK, and enzyme required for DNA double strand break repair by the nonhomologous end joining pathway. The fourth was poly (ADP-ribose) polymerase (PARP), an enzyme which is involved in numerous "caretaker" functions in the cell, guardian of genomic integrity. These findings were surprising, as none of these proteins are classical mediators of transcriptional proteins such as HOX, and lead to further investigation as to the functional relevance of these interactions.

We found that HOXB7 was a substrate for DNA-PK phosphorylation *in vitro* as well as ADP-ribosylation by PARP *in vitro* and *in vivo*. The target site for ADP-ribosylation was determined to be at the glutamic acid- rich carboxy terminus of HOXB7. When HOXB7-overexpressing cells were irradiated so as to cause DS DNA breaks, these cells repaired DNA faster than parental and control cells with higher overall survival rates. These cells also had better telomeric maintenance than controls, indicative of enhanced replicative potential. When tested for the ability to transform cells on soft agar growth assays, the HOXB7-overexpressing cells appeared to require PARP activity. We thus conclude that cells that breast cancer cells which overexpress HOXB7 have an enhanced survival advantage even after treatment with DNA damaging agents and that HOXB7-mediated transformation may require post-translational modifications such as ADP-ribosylation. We are currently examining if this mechanism involves competition with endogenous PARP substrates, such as centrosomal proteins, which become deprived of their post-translational modifications, disregulating their function, which leads to altered centrosomal copy numbers and aberrations in ploidy.

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